Characterization of Wax Esters, Triglycerides, and Free Fatty Acids of Follicular Casts

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The abnormal impactation of a sebaceous follicle (the follicular cast) has been implicated as the preclinical lesion of acne vulgaris. We have characterized the lipid composition of these structures in the first of a series of studies aimed at the identification of sebaceous lipids that may be associated and/or responsible for the initiation of clinical lesions. The lipid composition of follicular casts was analyzed using thin-layer chromatography, gas chromatography, and mass spectrometry. The mean wet weight of the casts was 24.7 ± 8.6 μg and 7.2 ± 5.6 μg (29.4 ± 13.5%) was lipid. Cholesterol (3.8 ± 1.8%) and cholesterol esters (2.0 ± 2.7%), wax esters (25.3 ± 6.0%), squalene (19.9 ± 6.6%), triglycerides (16.1 ± 7.8%), and free fatty acids (33.0 ± 10.0%) were all present in cast lipid. Fatty acids of the free fatty acid and triglyceride fraction ranged from C12 to C22. The major components of the free fatty acids were C14:0, C15:0, C16:1, C16:0, 2-me-C17:0, and C18:1. In the triglyceride fraction C14:0, C15:0, C16:0, C18:1, and C18:0 dominated. The free fatty acids were composed of normal saturated (50.6%), normal unsaturated (32.8%), and monomethyl branched (16.6%) acids; the triglyceride fraction contained (86.3%) normal saturated (10.8%), normal unsaturated, and (3.0%) monomethyl branched fatty acids. Wax esters of follicular casts included esters ranging from C26:1 to C38:0. Saturated esters predominated and both odd- and even-numbered esters were present. The most abundant fatty acid moieties of these esters were C16:0 and C15:0, whereas C14:0, C17:0, and C20:0 were the most frequently detected alcohol moieties. *J Invest Dermatol 86:700–705, 1986*

Abnormal sebum production, abnormal keratinization of the epithelium of sebaceous follicles, and inflammation generated by *Propionibacterium acnes* are all involved in the pathophysiology of acne vulgaris. A major drawback in the study of these factors is that only some of the follicles are involved in this mosaic of disorders at any one time, and unobtrusive sampling of individual sebaceous follicles has not been possible in the past. However, with the use of the cyanoacrylate follicular biopsy technique [1], ultrastructural studies of abnormal follicles have been carried out. Using the cyanoacrylate glue technique it is possible to remove the outer 2 or 3 cell layers of the epidermal stratum corneum to which are attached portions of the acro- and infrainfundibulum of sebaceous follicles. These follicular samples consist of vellus hairs in a matrix of sebum and bacteria and have been referred to as "coated hairs" [2], microcomedones [3], and "follicular casts" [4]. The term follicular cast is preferable as it is still unclear whether all of these structures evolve into comedones and inflammatory lesions. However, several features of these casts suggest that they should indeed be considered abnormal. First, the occurrence of lipid droplets in the horny cells of these structures has been demonstrated [5–7], and this has been considered a marker for abnormal keratinization in psoriasis [8,9] and pityriasis rubra pilaris [10]. Second, horny cells of follicular casts contain lamellar configurations, which are probably abnormal accumulations of polar lipids due to faulty lipid metabolism [5–7]. And third, horny cells closest to the sebum-filled lumen contain large lipid masses leading to the formation of "balloon-shaped" cells [4,11]. However, despite the ultrastructural observations on these casts, the preclinical events that initiate such changes in the sebaceous follicle and subsequently lead to the formation of a clinically visible lesion are as yet unclear.

The role of sebum in acne is poorly understood, and differences in the rate of sebum production in acne patients and normal controls have so far been the only consistent finding [12–14]. Several studies have attempted to study compositional differences of sebum in acne and normal skin, however these have not contributed much to the understanding of the effects of sebum on the sebaceous follicle. In many of these studies the lipid has been collected from a large area of the face or from the scalp of only a few subjects [15,16]. Because acne affects only a portion of follicles, the use of scalp lipid may not be appropriate as acne does not occur in this area. Some information on the composition of lipid of open and closed comedones is available [17–19], however these comedones may represent structures that are too far evolved to provide information on the initial changes that are occurring in the follicles.

We believe that the characterization of lipid from abnormal sebaceous follicles (follicular casts) may provide more detailed information on the possible role of sebum in acne vulgaris. We chose to study the composition of triglycerides (TG), free fatty acids (FFA), and wax esters (WE) in detail. FFA have in the past

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Abbreviations:
EI: electron impact
FAME: fatty acid methyl esters
FFA: free fatty acid(s)
GC-MS: gas chromatography-mass spectrometry
TG: triglyceride(s)
TLC: thin-layer chromatography
WE: wax ester(s)

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been thought to act as irritants in the acne lesion and therefore much data were accumulated on the composition and the possible role of the fatty acids and the TG from which they are released, [20–23]. However, several studies disputed the theory and it was shown that injection of fatty acids in physiologic doses into the skin did not produce acne lesions [24]. On the other hand, the concentration as well as the detailed composition of FFA and TG in the follicular casts are completely unknown and as fatty acids do possess comedogenic properties [22,23] we felt that these warrant some attention. Our interest in the wax esters is based on the finding that successful treatment of acne with 13-*cis*-retinoic acid results in a selective suppression of the wax esters in the skin surface lipid [25]. Furthermore, wax ester increases are found in the skin surface lipid film of children with early onset of acne [26,27].

MATERIALS AND METHODS

A total of 389 follicular casts were obtained from 20 adults with acne using the cyanoacrylate follicular biopsy technique [1]. Areas with inflammatory lesions were not sampled. The casts were dissected off the slides and weighed using a Cahn electrobalance model 27 (Cahn Instruments, Cerritos, California). Five casts each from 12 individuals were analyzed for total lipid composition, the rest of the material was pooled for GC (gas chromatography) and GC-MS (gas chromatography-mass spectrometry) studies. Lipid was extracted by sonication for 20 min on ice in 1 ml hexane. The extractions were filtered to remove bacterial and cellular debris and dried under a stream of nitrogen at 40°C. Samples were reconstituted in 200 μl hexane and separated into the major lipid classes using thin-layer chromatography (TLC). The lipids were spotted in 5-μl aliquots onto 250 μm, 20 cm × 20 cm, silica gel G TLC plates (Analtech Inc., Newark, Delaware) and developed according to the system of Downing [28]. The lipid spots were visualized by spraying with 75% H_2SO_4 and heating the plates to 220°C for 55 min on hotplates.

The charred chromatograms were then scanned using a dual-beam photodensitometer (Schoeffel Instruments Inc., Newark, New Jersey) and peak areas were integrated using a Spectraphysics SP 4100 computing integrator (Spectraphysics Inc., San Jose, California). The amount of each lipid was calculated by reference to the charring efficiency of known standards (Nu Check Prep, Elysian, Minnesota) [29].

The FFA, TG, and WE fractions were further characterized using GC and MS. These lipid fractions were isolated using TLC. The remainder of the samples was spotted in 20-μl aliquots onto the plates and developed as described above. The lipids were, however, visualized by exposure to iodine vapor in a closed chamber and scraped off the silica into acid-washed test tubes. The lipids were eluted off the silica with chloroform. The samples were filtered and dried.

TG were saponified with 1 ml 5% NaOH in 50% methanol for 15 min at 100°C. The samples were then cooled and acidified with 4 N HCl to pH 2.0. Fatty acids released by the saponification of TG and the FFA were converted to their methyl esters (FAME) by heating at 80°C for 5 min in 14% boron-trifluoride in methanol (Sigma Chemical Co., St. Louis, Missouri). After 1 ml of H_2O had been added, the FAME were extracted twice with 1 ml chloroform:hexane (1:4). After the FAME had been analyzed using GC-MS, they were converted into pyrrolidides [30] to establish the position of the double bond on the unsaturated fatty acid components. WE were not derivatized, but dried, reconstituted in hexane, and analyzed intact.

The GC-MS system was a Finnegan Model 4510 Quadrupole GC-MS system equipped with a Finnegan Incos 2300 data system (Finnegan MAT Inc. Sunnyvale, California). Each sample was injected into a 30 m × 0.32 mm CPSil-8 fused silica capillary column (Chrompack Inc., Bridgewater, New Jersey) using a splitless injection technique. The injector temperature for the FAME and the WE was 250°C. The sweep valve was closed for 45 s during injection. The temperature of the column oven for the FAME was programmed from 100°–300°C at 8°C/min and held at the final temperature for 10 min. For WE the temperature was programmed from 200°–300°C at 8°C/min, final hold 45 min. The separator temperature for all runs was 325°C. Electron impact (EI) mode of ionization was used for all samples with the electron energy set at 70 eV, the filament current at 0.3 mA, and the electron multiplier at 1450 V. FAME were scanned from mass 40–400 and WE from 40–650.

RESULTS

Total Lipid Composition The mean wet weight of the casts was 24.7 ± 8.6 μg and 7.2 ± 5.6 μg (29.4 ± 13.5%) was lipid (Table I).

Free Fatty Acids and Triglycerides of Follicular Casts Fatty acids from the FFA and TG fractions of the casts included

Table I. Lipid (%) Composition of Follicular Casts

Subject	CH	FA	TG	WE	CHE	SQ
1	1.1	28.2	16.7	29.3	6.3	18.4
2	6.5	25.4	5.0	33.3	7.5	22.4
3	4.0	46.4	9.6	27.2	1.6	11.2
4	4.7	36.5	12.4	32.9	0.0	13.5
5	5.0	32.0	32.0	18.6	0.0	11.9
6	3.2	25.8	9.0	28.6	3.2	30.0
7	2.5	18.0	18.8	27.5	3.8	29.4
8	1.4	22.2	24.6	47.4	1.7	22.8
9	2.3	41.9	9.3	23.3	0.0	23.3
10	3.1	31.3	21.9	18.8	0.0	25.0
11	5.4	51.5	13.5	13.5	0.0	16.2
12	6.0	25.8	9.0	28.6	3.2	30.0
Mean	3.8	33.0	16.1	25.3	2.0	19.9
SD	1.8	10.0	7.8	6.0	2.7	6.6

Key: CH = cholesterol
FA = fatty acids
TG = triglycerides
SD = standard deviation
WE = wax esters
CHE = cholesterol esters
SQ = squalene

Table II. Fatty Acid Composition of the Free Fatty Acid (FFA) and Triglyceride (TG) Fractions

Fatty Acid[a]	% in FFA	% in TG
C12:1	0.9	0
C12:0	0.9	tr[b]
C13:0	0.6	0
C14:1	1.8	tr
C14:0	10.6	12.8
C15:0 iso	1.4	0
C15:0 anteiso	3.0	0
C15:1	1.0	tr
C15:0	7.5	11.4
C16:1Δ6	15.0	tr
C16:0	23.4	51.7
2me-C17:0	8.1	1.3
C17:0 iso	4.1	1.7
C17:1	1.4	tr
C17:0	1.9	0
C18:1Δ8	8.7	8.1
C18:0	4.6	8.7
C20:1	0.9	2.7
C20:0	0.5	1.7
C21:1	1.7	0
C22:1	1.4	0
C22:0	0.6	0

[a]If not otherwise indicated, the fatty acid is of the normal series.
[b]Trace amounts.

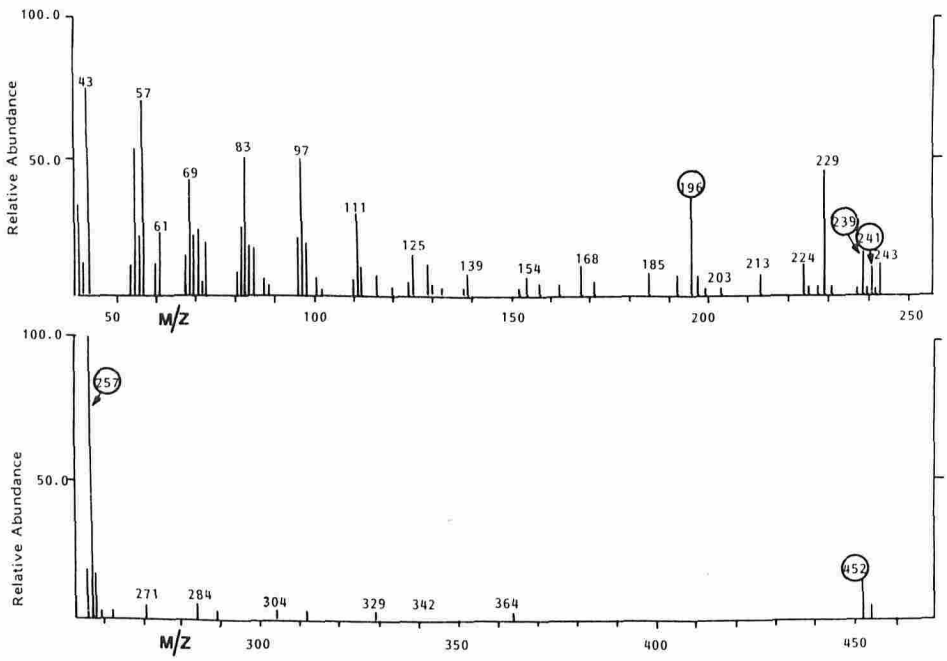

Figure 1. Mass spectrum of a C30:0 WE from follicular casts. Molecular ion m/z 452, fatty acid C16:0 m/z 239, 257; alcohol C14:0 m/z 196, 241. The small peak at m/z 224 is the $(R'-1)^+$ ion of a C16:0 alcohol and corresponds to the m/z 196 of the C14:0 alcohol. Similarly the large peak at m/z 229 is the $(RCO_2H_2)^+$ ion of the C14:0 acid corresponding to the m/z 257 of the C16:0 acid. Both the acids and the alcohols are most likely from the normal series as indicated by the absence of prominent peaks at m/z 56 and m/z 70.

chain lengths from C12 to C22 (Table II). The FFA fraction was composed of normal saturated (50.6%) and unsaturated (32.8%) acids, with branched acids contributing 16.6%. Saturated normal-chain fatty acids clearly dominated in the TG fraction (86.3%) with the normal unsaturated (10.8%) and the branched (3.0%) components contributing a lesser proportion. The branched acids were of the iso and anteiso type and one C17:0 fatty acid was identified as having a methyl branch at position 2 [31]. The position of unsaturation was located at Δ6 on the C16:1 fatty acids and on Δ8 on the C18:1 acids [30]. The position of the double bond for the other unsaturates could not be confirmed from the pyrrolidide derivatives due to low ion intensities. The most notable difference between the 2 fractions was the extremely low amount of C16:1 in the TG fraction. Furthermore the iso and anteiso C15:0 acids were not detected in the TG and the amount of 2me-C17:0 was substantially lower.

Wax Esters of Follicular Casts Due to the long chain length of WE, resolution by GC is not optimal. It is conceivable that 2 esters with the same molecular weight but different acid and alcohol components may elute at the same time. Therefore results for GC-MS studies will be emphasized. The molecular weight and thus the carbon number information for the WE was obtained from the molecular ion (MI). The constituent saturated fatty acids were identified by the mass of the $(RCO_2H_2)^+$ and $(RCO)^+$ ions, the alcohol component was deduced from the mass of the $(R'-H)^+$ and $(CO_2R')^+$ ions. The most characteristic ion for the unsaturated fatty acids was the abundant $(RCO-1)^+$. An example of a typical spectrum for the WE is shown in Fig 1. The type of branching was tentatively deduced from a high abundance of the ions m/z 56 and m/z 70 for iso- and anteiso compounds, respectively. Selective ion monitoring was also utilized for confirmation of identification. The WE of follicular casts contained members ranging from C26:1 to C38:0 (Table III, Fig 2). Saturated esters were detected more frequently than their unsaturated equivalents, and both odd and even chain lengths were present. Only one diunsaturated ester, C34:2, was detected; however the fragment ions were too low for identification of the acid and alcohol moieties. In our sample of follicular cast WE we detected several different acid and alcohol series for one particular ester. These WE with the same molecular weight, but a different composition eluted off with approximately the same retention time. The saturated fatty acids included C16:0, C15:0, C14:0, C17:0, C13:0,

Table III. Wax Esters of Follicular Casts

WE	MI	Acid	Alcohol
C26:1	394	NID	NID
C27:1	408	C16:0	C11:1
C27:0	410	C16:0	C11:0
C28:1	422	C16:1	C12:0
		C17:1	C11:0
C28:0	424	C16:0	C12:0
		C14:0	C14:0
C29:1	436	C16:0	C13:1
C29:0	438	C15:0	C14:0
C30:1	450	C16:1	C14:0
		C13:0	C17:1
C30:0	452	C16:0	C14:0
		C14:0	C16:0
C31:0	466	C14:0	C17:0
C31:0 ai	480	NID	NID
C32:1	478	C17:0	C15:1
		C17:1	C15:0
		C14:1	C18:0
C32:0	480	C16:0	C16:0
		C18:0	C14:0
		C15:0	C17:0
C33:1	492	C18:1	C15:0
C33:0	494	C16:0	C17:0
		C17:0	C16:0
		C15:0	C18:0
C33:1 ai	506	NID	NID
C34:0	508	C16:0	C18:0
		C17:0	C17:0
		C20:0	C14:0
		C14:0	C20:0
C34:2	504	C18:2	NID
C35:0	522	C16:0	C19:0
		C15:0	C20:0
C35:0 ai	536	NID	NID
C36:0	536	C16:0	C20:0
		C15:0	C21:0
C37:0	550	C17:0	C20:0
C37:0 i	564	NID	NID
C38:0	564	C16:0	C22:0

Key: WE = wax ester
MI = molecular ion
NID = not identified
i = iso
ai = anteiso

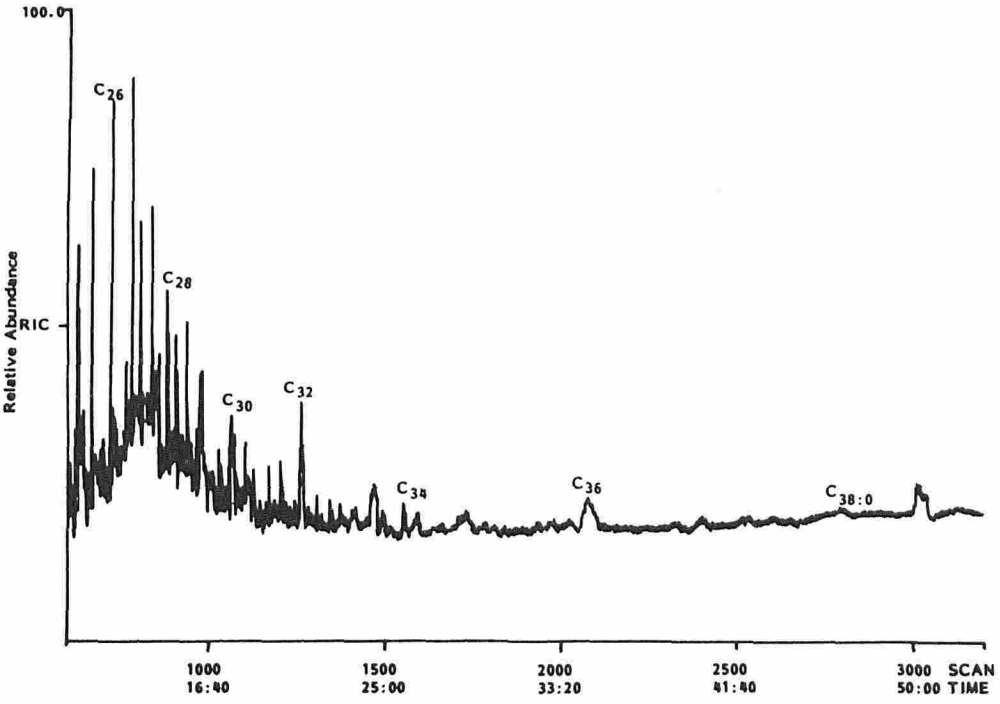

Figure 2. Reconstructed total ion chromatogram *(RIC)* of the WE of follicular casts: 200–300°C at 8°C/min; carrier gas He: 55 cm/s, 1 μl splitless injection.

C18:0, and C20:0 in decreasing frequency. Of the unsaturated fatty acids C16:1 and C17:1 were detected twice and C14:1, C18:1, and C18:2 once. The alcohols included a broader range of carbon chains: C14:0, C17:0, C20:0, C16:0, C18:0, C11:0, C12:0, C15:0, C19:0, C21:0, and C22:0 saturated alcohols were found in decreasing order. Four unsaturated alcohols were detected: C11:1, C13:1, C15:1, and C17:1.

DISCUSSION

The total lipid composition of follicular casts is very similar to that of skin surface lipid [13] except for the significantly higher percentage of FFA found in cast lipid. It is evident that hydrolysis of the TG primarily by microbes is much more complete in the casts as well as in open and closed comedones [17]. Therefore it is possible that the FFA do have a role in acne which has previously been undermined by studies that have been based on the analysis of surface lipids, where no significant differences have been demonstrated in the percentage of FFA of acne and normal skin. Further studies on the content of FFA in normal follicles are currently being pursued. On the other hand, the composition of the FFA and TG fractions of the follicular casts resembles that of comedones, which according to Nicolaides et al [17] is similar to that found on the skin surface.

We were able to detect some differences between the FFA and TG fractions. Both C15:0 iso and C15:0 anteiso acids were detected in the FFA but not in the TG and can thus be attributed to *P. acnes*, the main inhabitant of pilosebaceous follicles which characteristically contains C15 methyl branched fatty acids [32]. A C17:0 iso acid was detected in both fractions. This fatty acid was present in somewhat higher amounts in the FFA, which may indicate both a bacterial and/or sebaceous origin [32]. In addition, a 2me-C17:0 was present in both fractions, 8.1% in the FFA and 1.3% in the TG. The origin of the 2-me is open to speculation, a bacterial contribution is likely as methyl groups appearing at the 2 position for example in fatty acids of the vernix caseosa are low in amount if present at all [33]. Fatty acids of *P. acnes*, on the other hand, have only been characterized using packed column GC [32], and more sophisticated istrumentation may reveal a more complex lipid composition. It has been reported that the position of unsaturation on fatty acids can be deduced from their pyrrolidide derivatives [30]. We were able to confirm the position of the unsaturation for C16:1 (Δ6) and C18:1 (Δ8), however the other unsaturates were present in such low concentrations that characteristic ion intensities could not be observed. Furthermore, ion intensities for magnetic analyzers [30,34] and quadrupole analyzers are not necessarily comparable. Our data are in agreement with those of Nicolaides [35] who reported that C16:1Δ6 and C18:1Δ8 are the major fatty acid components of human sebum. Further experiments on confirmation of the location of the double bond are in process.

The range of WE in follicular casts that we have detected is shorter than that reported for comedones or for skin surface lipids, but the WE of the casts do appear to resemble comedonal esters. Nicolaides et al [17] showed that the WE of closed comedones range from C28 to C42, with C34, C36, and C38 as prominent peaks. However, in our studies on the follicular casts we have not detected esters longer than C38. This may be either a true difference or alternatively esters longer than C38 are below the limits of detection or obscured by background noise. We did not attempt to quantitate this pooled sample as it may not accurately reflect the proportions of the various peaks within an individual. Saturated and unsaturated esters and odd and even chain esters were found to occur in casts, and saturated esters were detected more frequently than the unsaturated esters. Nicolaides et al [17] similarly demonstrated that in comedones saturated chains predominated. Branched WE appear to occur in minor amounts in both follicular casts and in comedones. Harvey and Tiffany [36] have reported that iso- and anteiso branches may be identifiable by the characteristic high abundance of m/z 56 and m/z 70, respectively. However, we have designated our identification of the branched esters as "tentative." Although m/z 56 and m/z 70 were abundant in those esters designated as branched in Table III, undiagnostic ions were frequently seen in the spectra of these esters due to the amount of hydrocarbon in these compounds which complicated interpretation. Nicolaides et al [17] also found that the range of fatty acids in the WE includes C12 to C30 chains in the comedone, a range which is wider than is found in follicular casts. However, both in comedones and follicular casts C16:0 and C15:0 are 2 of the major components. The most abundant alcohol components of follicular casts were the C14:0, C17:0, and C20:0. Haahti [37] and Nicolaides [38] studied the composition of WE of skin surface lipid and demonstrated that the alcohol components include C20 chains. In follicular casts we detected even C21:0 and C22:0 alcohols.

Our GC-MS analysis of the intact WE of cast lipid is the first application of this very powerful analytical technique to the study of potential acne lesions. Aasen et al [39] have shown that EI mass spectra of WE obtained by direct insertion probe and a magnetic sector mass spectrometer contain the characteristic ions $(RCO\text{-}OR')^+$, $(RCO_2H_2)^+$, $(RCO)^+$, $(R'\text{-}H)^+$, and $(CO_2R')^+$. Our GC/quadrupole data are in agreement, however, the most intense and hence the most useful fragment ions were $(RCO_2H_2)^+$, $(RCO)^+$, and $(R'\text{-}H)^+$. Furthermore, as has been demonstrated by Spencer [40] one of the most diagnostic ions for the unsaturated fatty acids is the $(RCO\text{-}1)^+$ ion. The $(R'\text{-}H)^+$ ions tended to be weak, in particular when unsaturation was present in the alcohol moiety. This is due to the fact that the ionic charge is preferentially retained by the resonance-stabilized $(RCO_2H_2)^+$ ion. Hydrolysis of the ester and derivatization of the acid and the alcohol [36] would also provide important information on the detailed structural composition of the component acid and alcohols. However, in this study we wished to study the intact esters and to determine the total chain lengths and the major components. A definite advantage of MS is the ability to detect and exclude from the interpretation of data any possible contaminating cholesterol esters that may originate from incomplete separation of wax and cholesterol esters on TLC. Routinely these may be separated on plates or on columns containing magnesium oxide or magnesium hydroxide [41,42]. With small samples, recovery of the wax ester may be severely affected by such preparative steps. We have not detected interfering cholesterol esters, probably due to the fact that they occur in one-tenth the amount of the WE.

We have demonstrated that some 29% of the wet weight of follicular casts is lipid which is composed of cholesterol and its esters, WE, squalene, TG, and FFA. The hydrolysis of TG in casts is much higher than on the skin surface, a finding that may be of significance with reference to acne vulgaris. The FFA and TG fractions of follicular casts ranged from C12 to C22, and were similar to those of comedones. The FFA are derived both from sebum and the resident bacteria. The WE of follicular casts range from C26 to C38, the most abundant fatty acid components being C16:0 and C15:0, with C14:0, C17:0, and C20:0 being the major alcohol moieties. To assess the relevance of our findings we are currently studying follicular casts from a number of subjects and also the lipid composition of normal follicles and these findings will be the subject of a further communication.

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